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GASTRULATION IN THE TURBELLARIAN *HYDROLIMAX GRISEA* (PLATYHELMINTHES; PLAGIOSTOMIDAE): FORMATION OF THE EPIDERMAL CAVITY, INVERSION AND EPIBOLY[1]

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Gastrulation of the commensal turbellarian, *Paravortex gemellipara*, was defined by Ball (1916, p. 507) in the statement: "If by the process of gastrulation is meant the infolding or overgrowth of entoderm by ectoderm, then true gastrulation occurs. . . ." Ball's definition covers all processes, direct or indirect, by which ectoderm achieves its definitive position in the developing turbellarian. Entolecithal embryos of acoels and of polyclads gastrulate by epiboly or overgrowth by micromeres of yolk-bearing macromeres and their derivatives (Bresslau, 1933; Kato, 1940, 1968). Ectolecithal embryos of rhabdocoels, alloeocoels and triclads gastrulate in various ways, all of which may well represent adaptations to or modifications by the yolk cells which surround the developing embryo (Kato, 1968).

According to Ball (1916) the first step in the gastrulation of the ectolecithal embryo of *P. gemellipara* is the differentiation of the ectoderm. The outer cells of the embryonic mass become flattened and stretch posteriorly to entrap the endodermal cells which have enveloped the yolk cells. Ball concluded that gastrulation of *P. gemellipara* occurs by epiboly. Bresslau (1904) had earlier observed a similar process in *Mesostoma*. As cited by Hyman (1951, p. 175), Bresslau observed that the peripheral cells of the venter of the embryo of *Mesostoma* ". . . arrange into a surface epithelium, which is the ventral epidermis and which gradually spreads dorsally to enclose the dorsal yolk mass." The gastrulation of *Monocelis fusca* is accomplished by the differentiation and spreading of eight blastomeres (six *Hüllzellen*, two *Vitellocytophagen*), which form a peripheral embryonic epithelium (Giesa, 1966). The *Vitellocytophagen*, at the vegetal pole, engulf yolk cells, and are subsequently overgrown by four spreading *äquatorialen Hüllzellen*. The vegetal *Hüllzellen* and additional *Hilfzellen* transfer the remaining yolk cells into the body of the embryo. The definitive epidermis of *M. fusca* is formed when blastomeres move into the embryonic epithelium from the embryonic mass. The blastomeres differentiate into the epidermal cells, the nuclei of which sink back into the parenchyma (embryonic mass) of the turbellarian.

Gastrulation of triclad embryos involves the formation of transitory structures which do not contribute to the definitive organism (Bresslau, 1933; Kato, 1968). Blastomeres form a thin outer provisional ectoderm around the central yolk syncytium after the yolk cells aggregate. At one point on this provisional

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ectodermal sac a transitory pharynx forms by accumulation of blastomeres. A thin-walled cavity forms at the inner end of the temporary pharynx. The cavity is lined by a thin layer of cells, the internal membrane. The external cellular yolk cells are sucked through the transitory pharynx into the thin-walled cavity, now termed the temporary intestine. Between the wall of the temporary intestine and the provisional ectoderm, the definitive epidermis differentiates from proliferating blastomeres and spreads to replace the degenerating cells of the provisional outer and inner membranes.

A remarkable process of gastrulation has been observed in a series of studies on the developmental cycle of *Hydrolimax grisea*. *Hydrolimax* is the only freshwater member of the alloeocoel family Plagiostomidae reported from North America (Hyman, 1938). First described by Haldeman (1843), *Hydrolimax* was rediscovered by Hyman (1938) in collaboration with Ulric Dahlgren of

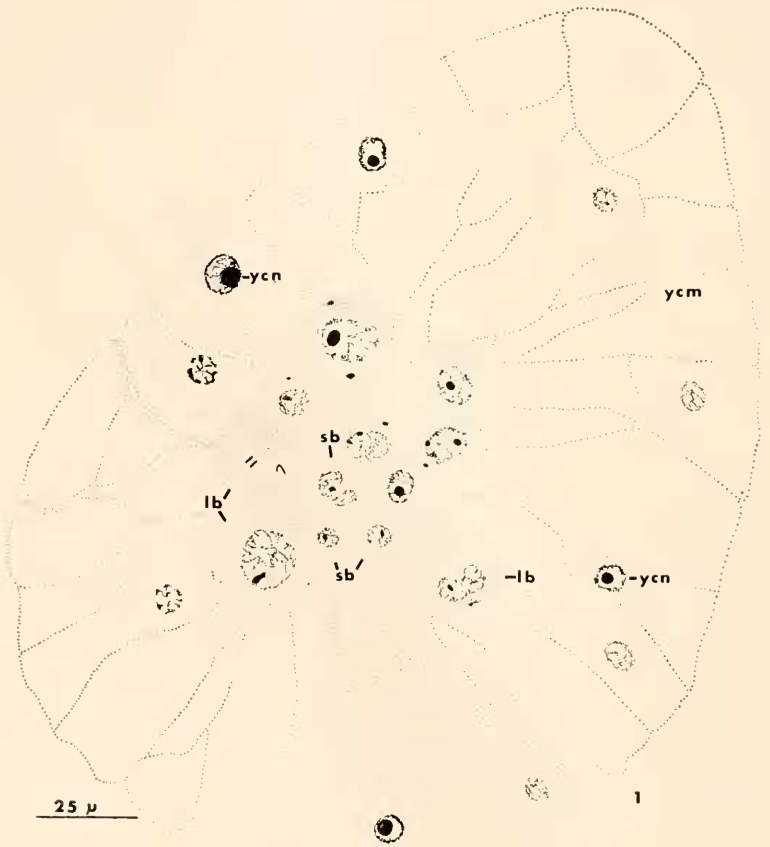

FIGURE 1. Cleaving embryo of *Hydrolimax grisea* enveloped by radially arranged (and closely adhering) yolk cells. Note the arrangement of the blastomeres: the smaller blastomeres are situated in the center of the embryo (embryonic mass); Costello's fixative, 4° C; lb, large blastomere; sb, small blastomere; ycm, yolk-cell mass; ycn, yolk-cell nucleus.

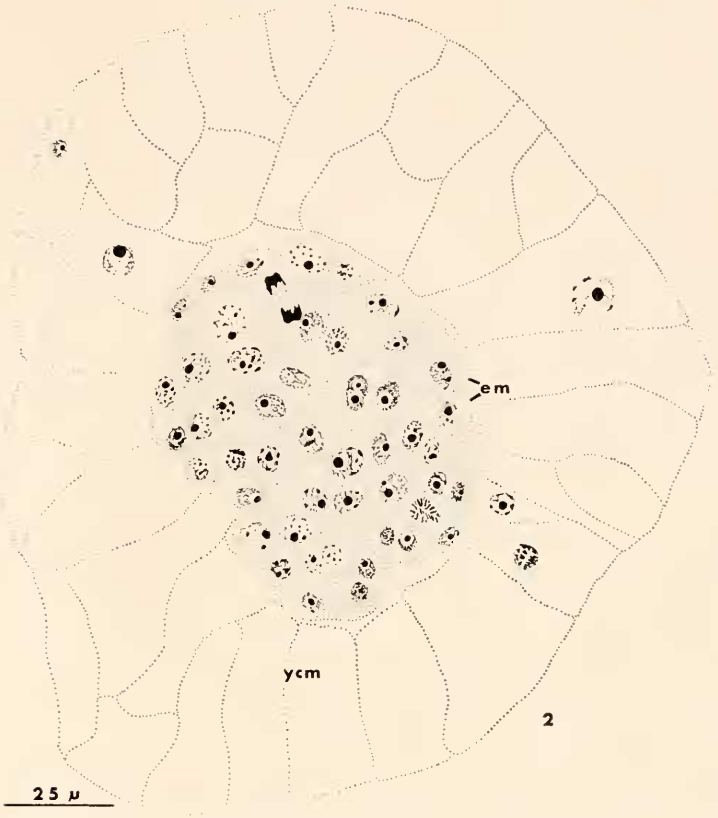

FIGURE 2. Older embryo: a solid mass of blastomeres situated in the center of the yolk-cell mass. Slightly older embryos are bilobed; Heath's polyclad fixative, 4° C; em, embryonic mass.

Princeton University. Hyman (1938) gave an account of the morphology and taxonomy and reviewed the scant literature of this turbellarian. A review of the more recent literature and a study of the oögenesis of *Hydrolimax* is presented in an earlier paper (Newton, 1970).

MATERIAL AND METHODS

The cocoons of *Hydrolimax* are characteristically red-brown in color and surprisingly large (up to 2 mm in diameter) for the size of the adult turbellarian (4 to 10 mm in length; 1 to 2 mm in width), as noted by Hyman (1938). They are deposited on hard surfaces, the underside of rocks, leaves, on bark of submerged tree trunks and rarely on emergent plants. The cocoons are deposited in the early spring, late March or mid-April, to June.

The material for this study was collected from two sources, Little Creek, east of Chapel Hill, and Morgan Creek, below University Lake impoundment west of Chapel Hill, North Carolina. Prior to fixation the cocoon capsules were

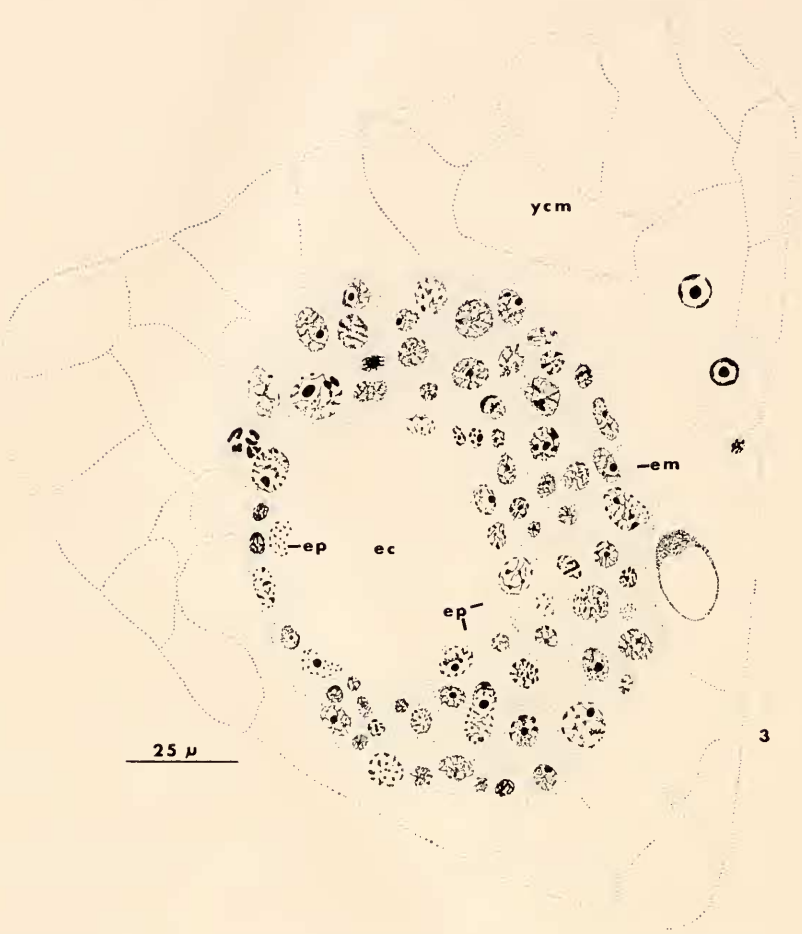

FIGURE 3. Embryo with expanding epidermal cavity lined by lightly-staining epidermal cells. The embryo is approaching the surface of the yolk-cell mass; Worcester's fixative, 25° C; ec, epidermal cavity; ep, epidermis.

punctured with insect pins to permit penetration of fixatives, as the capsule proved impermeable to the fixatives. During washing, the cocoons were opened up more to aid in washing, dehydration and infiltration. The specimens were placed in stender dishes and flooded with one of the following fixatives: Bouin's, Allen's, Worcester's, Heath's polyclad fixative and D. P. Costello's modification of Heath's fluid (sat. mercuric chloride, 81 parts; formalin, C. P., 9 parts; glacial acetic acid, 5 parts). The fixatives were used at initial temperatures of 25° C and 4° C. In addition some material was fixed in Bouin's at 37° C. The cocoons remained in the fixatives from 4 to 12 hours. Material fixed in Bouin's and Allen's fluids was washed in 70% ethanol until picric acid no longer leached out. Material fixed in Worcester's, Heath's and Costello's fluids was washed in several changes of distilled water for a period slightly longer than the time of fixation.

The cocoons were dehydrated, cleared, and infiltrated and embedded in filtered Paraplast (M. P. 56–57° C). Serial sections, at 8 μ, were cut. The sectioned material was stained with Heidenhain's iron-haematoxylin according to the procedures and recommendations of McClung and Conn (1937). Material fixed in Bouin's and Allen's fluids was more difficult to extract, taking longer, and, in the final stages of extraction, more difficult to control. Drawings were made with the aid of a camera lucida attached to a Spencer student microscope with a 4 mm objective and 15 × ocular.

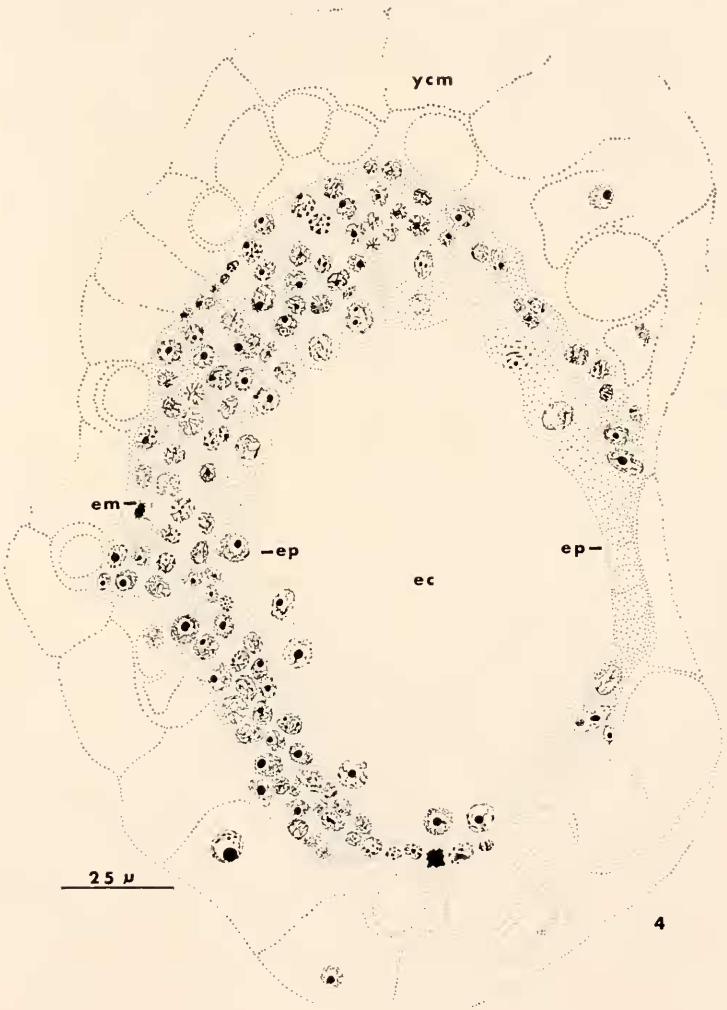

FIGURE 4. Epidermal cavity has expanded, forcing the overlying yolk cells aside. The epidermis nearest the surface of the yolk-cell mass will thin and become perforated. Note increased number of epidermal cells; Worcester's fixative, 25° C.

Some shattering of sections by cocoon capsule fragments did occur, but, in general, excellent serial sections were obtained.

Observations

Hydrolimax grisea, an hermaphroditic animal, practices mutual insemination by injection of spermatozoa through the wall of the genital atrium into the adjacent parenchyma (Hyman, 1938; Kepner, Stirewalt and Ferguson, 1941; Stirewalt, Ferguson and Kepner, 1942). The spermatozoa make their way through

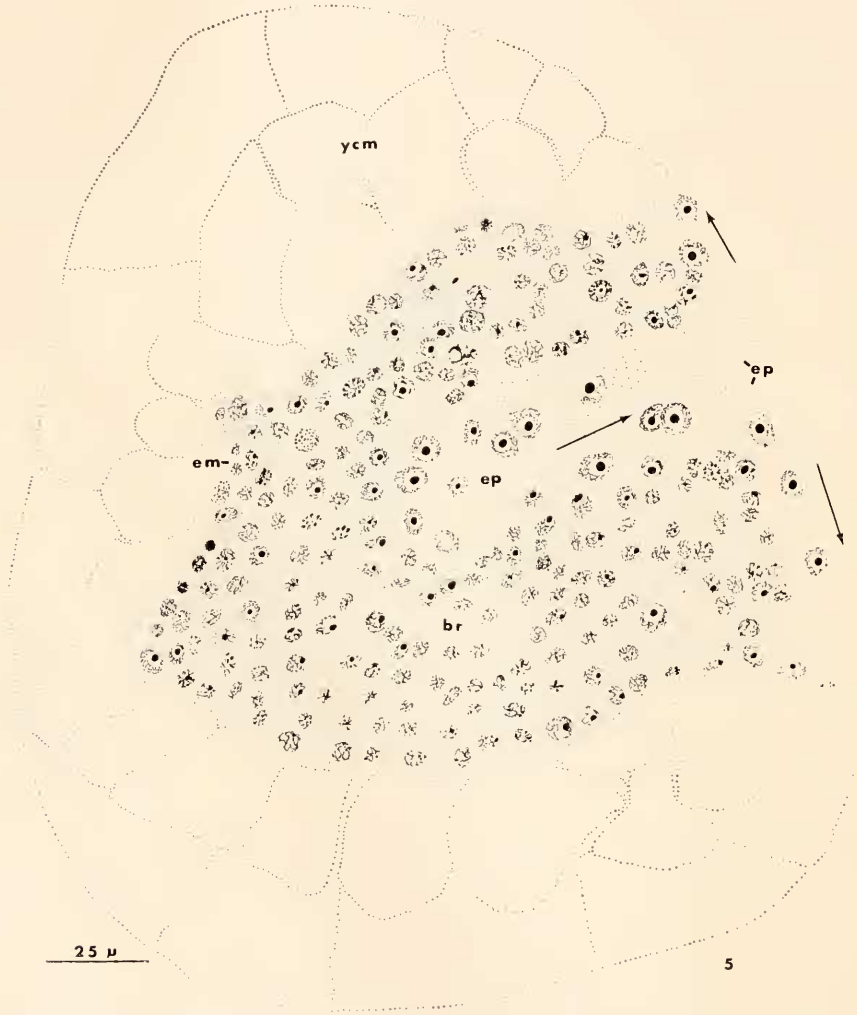

Figure 5. Epidermal cavity has collapsed and the embryo is in the process of inversion. The embryo rises out of and spreads over the yolk-cell mass (arrows); Worcester's fixative, 25° C; br, brain.

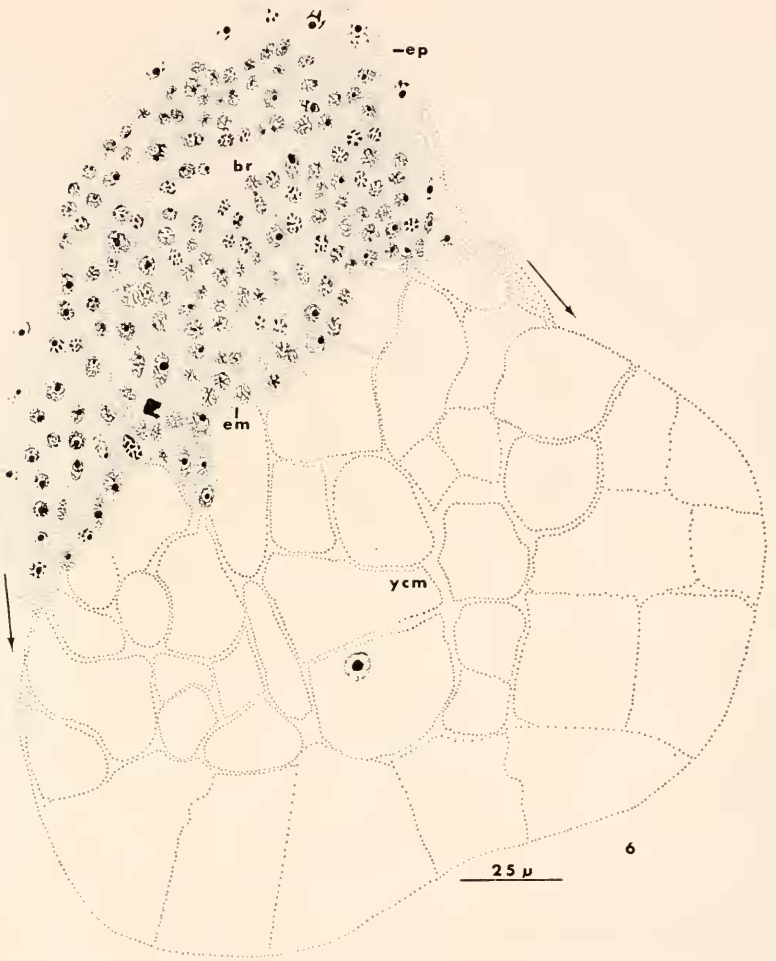

FIGURE 6. Inverted embryo of *Hydrolimax*. The lightly-staining epidermis is spreading posteriorly to entrap the yolk-cell mass; Bouin's fixative, 25° C.

the parenchyma of the adult turbellarian to the oöcytes (Hyman, 1938). At no time do the spermatozoa enter into the oviduct, as I have observed in studies of many sectioned specimens of mature *Hydrolimax*. As described earlier (Newton, 1970), the oöcytes are fertilized prior to breakdown of the germinal vesicle. The fertilized oöcytes migrate to the common genital atrium where 9 to 36 of them are incorporated, along with many yolk cells, into the cocoon. At the time of cocoon formation, the oöcytes are in meiotic prophase I (Newton, 1970).

Prior to the maturation divisions of the eggs, the yolk cells are spherical and loosely arranged around the oöcytes. By meiotic metaphase I, the yolk cells elongate and become radially arranged around the egg. They adhere closely to the egg and maintain a closed envelope around the developing embryo (Figs.

1–5). Cleavage of the egg occurs within this enveloping yolk-cell mass. The yolk cells follow or push into the cleavage furrows, apparently causing the wide separation of the blastomeres. In the late cleavage stages of the embryo of *Hydrolimax* a pattern of cell distribution emerges. The blastomeres, at first scattered among the yolk cells, aggregate toward the center of the yolk-cell mass, with the smaller blastomeres oriented toward the center of the embryo (Fig. 1). Cleavage continues until the embryo is a solid mass of cells (Fig. 2). In embryos slightly older than that of Figure 2 the mass of blastomeres, the main embryonic mass, is bilobed.

The cytoplasm of the blastomeres in the center of the embryo becomes less basophilic than the cytoplasm of surrounding blastomeres. A cavity appears among these lightly-staining cells and expands (Fig. 3). The cells which line the expanding cavity are derived from the lightly-staining blastomeres. *These cells lining the cavity are the epidermal (ectodermal) cells of Hydrolimax.* At stages of development slightly later than that of Figure 4, the cells lining the cavity are, at the light microscope level, similar in many respects to the epidermal cells of the adult *Hydrolimax*. It is this similarity which first drew my attention to the origin of the epidermis of this turbellarian. Because the cavity is lined by epidermis, it will be called the *epidermal cavity*. The epidermal cells flatten and spread as the cavity continues to expand. They increase in number, apparently through recruitment from the main embryonic mass, as no mitoses are seen among the epidermal cells.

The embryo approaches the surface of the enveloping yolk cell mass (Fig. 4). At this stage the embryo may be described as a mass of cells to one side of which is the expanding epidermal cavity. In Figure 4 the epidermal cavity is close to the surface of the yolk-cell mass and the main embryonic mass is centrally disposed. That portion of the embryo near the surface of the yolk-cell mass consists of a single layer of epidermis with a few associated embryonic cells. The covering yolk cells withdraw or are pushed aside by the expanding epidermal cavity. The epidermis thins and becomes perforated; the opening which appears unites the epidermal cavity with the environment within the cocoon. The epidermal cavity becomes obliterated by compression of the embryo by other embryos and yolk-cell masses within the cocoon (Fig. 5).

The epidermal cells spread out from the opening of the collapsed epidermal cavity as the embryo rises out of the yolk-cell mass. The embryo is thus completely inverted and comes to lie to one side of the yolk-cell mass (Fig. 6). During inversion and afterward, the epidermis, accompanied by a few internal embryonic cells, stretches posteriorly to cover and entrap the yolk-cell mass. Gastrulation in *Hydrolimax* is thus completed. At the close of gastrulation the embryo is bounded by a single layer of epidermal cells, beneath which are scattered the embryonic cells which accompanied the epidermis during epiboly. The anterior end of the embryo contains the main mass of embryonic (parenchymal) cells in which the brain (*cf.* Figs. 5 and 6) and pharynx are differentiating and from which will develop other definitive organs of the adult.

Discussion

The gastrulation of *Hydrolimax*, as described, is apparently unique. Compared with the gastrulation of other turbellarians, however, it parallels a two-part pattern of (1) differentiation of the epidermis and (2) movement of the

epidermis to its definitive location (*cf.* Ball, 1916). The differentiation of the epidermis of *H. grisea* is manifested by a loss of basophilia by the ectodermal cells in the center of the embryonic mass and by the formation of the epidermal cavity among the epidermal cells.

Unique to *H. grisea* is the differentiation of the epidermal cells in the center of the embryo. In *Paravortex* (Ball, 1916) and in *Mesostoma* (Bresslau, 1904) the epidermal cells appear on one side of the embryonic mass, *after* the embryo has moved to one side of the yolk-cell mass. They flatten and spread, entrapping yolk cells and parenchymal cells. Bresslau's (1904) study of the embryonic development of *Plagiostomum girardi* revealed an interesting pattern of gastrulation as related to the early cleavage and distribution of blastomeres: (1) The micromeres are always directed toward the capsule wall of the cocoon. (2) The epidermis arises on the side of the embryo facing the capsule wall. The prospective epidermal cells migrate to the surface of the yolk-cell mass and differentiate, flattening and spreading. The central disposition of the small blastomeres of the embryo of *Hydrolimax* suggests a similar pattern: The small blastomeres are directed toward the center of the embryo. The epidermis arises in the center of the embryonic mass. However, studies of the lineage of the epidermal cells of *Hydrolimax* are incomplete.

In *Hydrolimax,* the formation and expansion of the epidermal cavity are the initial steps of gastrulation. The epidermal cavity is not a blastocoel (*cf.* Giesa, 1966). The expansion of the epidermal cavity apparently pushes the yolk cells aside and forces the epidermal cells to the outer surface of the yolk-cell mass where they can participate in the subsequent steps of gastrulation, inversion and epiboly. Inversion in the case of *Hydrolimax* is accomplished when the embryo, displaced from the center of the yolk-cell mass, opens out onto the surface of the yolk-cell mass. The inversion as described for *Hydrolimax* is not comparable to "inversion" as it occurs in certain sponge larvae: the flagellated choanocytes invert through the osculum of the larval sponge, forming an amphiblastula. The choanocytes assume their definitive position within the body of the sponge during gastrulation by a process of invagination (Okada, 1968).

The process of gastrulation of the embryo of *Hydrolimax* can perhaps be more appreciated and better understood by considering the environment in which the embryo develops: as a pre-meiotic oöcyte, and during meiosis, cleavage, and early gastrulation, the developing turbellarian is surrounded by yolk cells which contribute nothing directly to the definitive organs. The yolk cells provide nutriment to the embryo and to the juvenile turbellarian after it emerges from the cocoon. Each embryo in the cocoon is surrounded by its own yolk-cell mass. The yolk cells adhere closely to the egg. They push into the cleavage furrows and apparently cause or contribute to the initial scattering of the blastomeres through the yolk-cell mass. The blastomeres reaggregate within the center of the yolk-cell mass—by what process or force remains to be discovered. There the blastomeres organize and/or differentiate for the task of inversion, which is accomplished to a large extent by the expanding epidermal cavity.

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Summary

1. Three events characterize the gastrulation of *Hydrolimax grisea*: formation of the epidermal cavity, inversion, and epiboly.

2. The epidermal cavity, the outstanding feature of gastrulation in this animal, forms in the center of the embryo among the lightly-staining cells of the future epidermis. The cavity expands, pushing aside the yolk cells which surround the embryo. Eventually the cavity becomes open at the surface of the yolk-cell mass. It is subsequently obliterated by compression from the other embryos within the cocoon.

3. Inversion begins when epidermal cells, which lined the epidermal cavity, spread around the yolk-cell mass from the opening of the collapsed cavity. The embryonic mass rises from the center to the surface of the yolk-cell mass, contributing to the progress of the inversion.

4. When the embryo is located to one side of the yolk-cell mass, epiboly begins. The epidermal cells stretch posteriorly to entrap the yolk-cell mass. A few parenchymal cells accompany the spreading epidermis.

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